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Structure of carbohydrate unit A of porcine thyroglobulin

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The unit A-type glycopeptides were purified from porcine thyroglobulin by Pronase digestion followed by chromatography on a DEAE-Sephadex A-25 column. These glycopeptides were separated into five fractions (UA-I, -II, -III, -IV and -V) by Dowex 50W (X2) column chromatography. Fractions UA-I, -II, -III, -IV and -V were found to have the compositions $(Man)_9(GlcNAc)_2$-Asn, $(Man)_8(GlcNAc)_2$-Asn, $(Man)_7(GlcNAc)_2$-Asn, $(Man)_6(GlcNAc)_2$-Asn and $(Man)_5(GlcNAc)_2$-Asn respectively. The structures of these five fractions were investigated by the combination of exo- and endo-glycosidase digestions, methylation analysis, Smith periodate degradation and acetolysis. The results showed that fraction UA-V had the simplest structure:

$$\begin{array}{l} \text{Man}\alpha 1\text{–}6\text{Man}\alpha 1\text{–}6\text{Man}\beta 1\text{–}4\text{GlcNAc}\beta 1\text{–}4\text{GlcNAc–Asn} \\ \quad\quad 3 \quad\quad\quad 3 \\ \quad\quad | \quad\quad\quad | \\ \quad\quad 1 \quad\quad\quad 1 \\ \text{Man}\alpha \quad\quad \text{Man}\alpha \end{array}$$

The larger glycopeptides (fractions UA-I, -II, -III and -IV) contained additional mannose residues $\alpha(1\rightarrow 2)$-linked to the terminal mannose residues in the above core structure. These unit A-type glycopeptides appear to be biosynthetic intermediates that are to be processed to form complex-type glycopeptides (unit B-type sugar chains).

The carbohydrate moiety of thyroglobulin has been extensively investigated with respect to its structure and biosynthesis. Thyroglobulin contains both high-mannose-type (unit A-type) and complex-type (unit B-type) oligosaccharides as asparagine-linked sugar chains in all species studied (Fukuda & Egami, 1971; Arima *et al.*, 1972). Recent studies suggested that high-mannose-type sugar chains were biosynthetic intermediates of complex-type sugar chains (Turco *et al.*, 1977; Tabas *et al.*, 1978; Hunt *et al.*, 1978). We were therefore interested in the structure of high-mannose-type sugar chains that attached to porcine thyroglobulin as precursors of its complex-type sugar chains, the structures of which are the subject of the accompanying paper (Yamamoto *et al.*, 1981). Ito *et al.* (1977) previously elucidated the structure of a major unit A-type glycopeptide from calf thyroglobulin. In the present study, we obtained five distinct unit A-type glycopeptide fractions, and found that they consisted of various high-mannose-type sugar chains having different numbers of mannose residues.

Abbreviations used: GlcNAcol, *N*-acetylglucosaminitol; XylNAcol, *N*-acetylxylosaminitol.

Materials and methods

Analytical procedures

Amino acids and amino sugars were determined with an amino acid analyser (Hitachi model 835; Hitachi, Tokyo, Japan) after hydrolysis in 6M-HCl at 100°C for 16h and in 4M-HCl at 100°C for 4h respectively. Neutral sugars were determined by g.l.c. on columns of 0.05% ECNSS-M (0.3cm × 100cm) and 3% OV-225 (0.3cm × 200cm), after conversion into alditol acetates by the method of Spiro (1972), hydrolysis for this assay being performed with 0.5M-H_2SO_4 at 100°C for 6h or with 2M-HCl at 100°C for 3h. Total neutral sugars and sialic acids were assayed by the phenol/H_2SO_4 reaction (Dubois *et al.*, 1956) and by the $NaIO_4$/

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resorcinol method (Jourdian *et al.*, 1971) respectively. Radioactive sugars eluted from columns were monitored with a liquid-scintillation spectrometer (Aloka LSC-700; Aloka, Tokyo, Japan).

Preparation of unit A-type glycopeptides from porcine thyroglobulin

Porcine thyroglobulin was prepared by the method of Ui & Tarutani (1961). After exhaustive Pronase digestion of the porcine thyroglobulin by the method of Fukuda & Egami (1971), unit A-type glycopeptides were purified by subjecting the resulting glycopeptide fraction to chromatography on a column (1.5 cm × 80 cm) of DEAE-Sephadex A-25. The column was eluted with 2 mM/1 mM-pyridine/acetate buffer. The unit A-type glycopeptides, which were eluted without adsorption, were further fractionated by chromatography on a Dowex 50 W (X2) column (2 cm × 150 cm). The column was equilibrated with 1 mM-pyridine/acetate buffer, pH 2.7, and the glycopeptides were eluted with the same buffer.

Radioisotopic labelling of unit A-type glycopeptides

Unit A-type glycopeptides were *N*-acetylated with [^{14}C]acetic anhydride (sp. radioactivity 30 Ci/mol, The Radiochemical Centre, Amersham, Bucks., U.K.) in 0.5% $NaHCO_3$ by the method of Ito *et al.* (1977), and then the radioactive glycopeptides were isolated by gel chromatography on a small column of Sephadex G-25.

Paper electrophoresis

Paper-electrophoretic analyses of [^{14}C]acetylated glycopeptides were carried out on Whatman no. 1 paper with pyridine/acetic acid/water (3:1:387, by vol.), pH 5.4, as a buffer, by the method of Tai *et al.* (1975). The electrophoretograms were obtained by scanning the papers with a model 7220 Packard Radiochromatogram Scanner.

Action of endo-β-N-acetylglucosaminidase H on unit A-type glycopeptides

Endo-β-*N*-acetylglucosaminidase H from *Streptomyces griseus* was purchased from Seikagaku Kogyo (Tokyo, Japan). *N*-[^{14}C]Acetylated glycopeptides (5×10^3 c.p.m., approx. 0.2 nmol) were incubated with 5 m-units of endo-β-*N*-acetylglucosaminidase H in 50 μl of 0.15 M-sodium citrate/phosphate buffer, pH 5.0, at 37°C for 24 h. For the digestion of unlabelled glycopeptides (0.15–0.2 μmol), 20 m-units of the enzyme was used in the same solution. Then the products were isolated as described by Yamashita *et al.* (1978).

Gel-permeation chromatography

Gel-permeation chromatography on a Bio-Gel P-4 column (0.3 cm × 100 cm) was performed with a high-pressure liquid chromatograph (Tri-rotor; Japan Spectroscopic Co., Tokyo, Japan) as described previously (Tsuji *et al.*, 1980).

Exoglycosidase treatment of ^{3}H-labelled oligosaccharides

α-Mannosidase was purifed from jack-bean meal by the method of Li & Li (1972). β-Mannosidase from snail (Sugahara & Yamashina, 1972) was kindly supplied by Dr. T. Okuyama, Seikagaku Kogyo. NaB^3H_4-reduced oligosaccharides (2×10^4 c.p.m.; approx. 1 nmol) were digested at 37°C with α-mannosidase (1.2 units) in 50 μl of 0.05 M-sodium acetate buffer (pH 4.0) for 16 h, then with β-mannosidase (0.1 units) in 30 μl of 0.1 M-sodium citrate buffer (pH 5.0) for 40 h. The product of each step was analysed by gel-permeation chromatography on a Bio-Gel P-4 column.

Methylation analysis

Methylation analysis of the glycopeptides and the products of Smith periodate degradation or acetolysis were performed by the method of Hakomori (1964).

Smith periodate degradation

Smith periodate degradation of $NaBH_4$-reduced oligosaccharides was performed as described previously (Irimura *et al.*, 1980).

Acetolysis

Acetolysis of the $NaBH_4$-reduced oligosaccharides was carried out by the method of Kocourek & Ballou (1969). The oligosaccharide alcohols (80–100 nmol) were then acetylated with a mixture of acetic anhydride (50 μl) and pyridine (50 μl) at 80°C for 6 h. The products were freed from the reagents by repeated evaporation with toluene, and dissolved in 0.2 ml of a mixture of acetic anhydride, acetic acid and H_2SO_4 (10:10:1, by vol.). Then the reaction was performed at 37°C for 16 h. To the reaction mixture pyridine (25 μl) was added and the solution was freed from solvent by repeated evaporation with toluene. After addition of 1 ml of water, the products were extracted with 1 ml of chloroform three times. The chloroform extracts were combined and then washed with 2 ml of water. After dehydration with anhydrous Na_2SO_4 and evaporation of the solvent, the acetolysate was deacetylated in 0.6 ml of methanol/water (9:1, v/v) containing 0.1% CH_3ONa at 25°C for 30 min. Then the resulting oligosaccharides were reduced by addition of 0.2 ml of 1 M-sodium acetate containing 0.5 M-NaB^2H_4. After incubation at 25°C for 2 h the reaction was terminated by addition of 0.1 ml of acetic acid, and the reaction mixture was passed through small columns of Dowex 50 W (X8; H^+ form) and Bio-Rad AG-1 (acetate form). The

remaining boric acid and acetic acid were removed by evaporation with methanol. The products thus obtained were analysed by gel-permeation chromatography on a Bio-Gel P-4 column.

Results

Isolation and characterization of unit A-type glycopeptides from porcine thyroglobulin

When the glycopeptide mixture from porcine thyroglobulin was applied to a column of DEAE-Sephadex A-25, unit A-type glycopeptides were recovered without adsorption, which were found to be free of sialic acids. Fractionation of the unit A-type glycopeptides on a Dowex 50W (X2) column gave five glycopeptide fractions (UA-I–UA-V) as shown in Fig. 1. Since some peaks overlapped slightly, fractions UA-I–UA-III were rechromatographed on the same column to ensure that each glycopeptide was pure.

N-[^{14}C]Acetylated fractions UA-I–UA-V were subjected to paper electrophoresis to check their purity. When the glycopeptides were monitored by radioactivity, each glycopeptide gave a single peak as shown in Fig. 2. Furthermore, the radioelectrophoretogram revealed that fractions UA-IV and UA-V co-migrated with ovalbumin glycopeptides IV and V respectively (Tai *et al.*, 1975). Fractions UA-I–UA-III were found to have slightly lower mobilities, suggesting that they were composed of larger sugar chains.

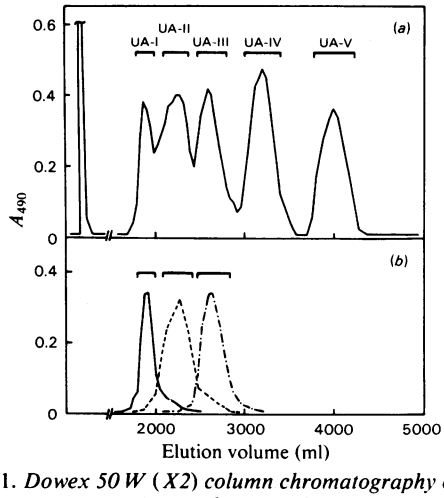

Fig. 1. *Dowex 50W (X2) column chromatography of unit A-type glycopeptides*

(*a*) The unit A-type glycopeptides were applied to a column (2cm × 150cm) of Dowex 50W (X2) equilibrated with 1mM-pyridine/acetate, pH 2.7, and elution was performed with the same buffer. Neutral sugars were analysed. (*b*) Fractions UA-I–UA-III were separately pooled and rechromatographed under the same conditions as described above.

Chemical composition analyses (Table 1) revealed that fractions UA-I–UA-V were so-called high-mannose-type glycopeptides, whose compositions were $(Man)_9(GlcNAc)_2Asn$, $(Man)_8(GlcNAc)_2Asn$, $(Man)_7(GlcNAc)_2Asn$, $(Man)_6(GlcNAc)_2Asn$ and $(Man)_5(GlcNAc)_2Asn$ respectively. To obtain a general idea of the structure of these glycopeptides, methylation analyses were performed. The results shown in Table 2 indicate that all five glycopeptides contained 3mol of non-reducing terminal mannose, 2mol of disubstituted mannose (at C-3 and C-6) and 2mol of C-4-substituted *N*-acetylglucosamine, and that none of the *N*-acetylglucosamine residues constituted a terminal residue. Fractions UA-I–UA-IV also contained 4, 3, 2 and 1mol of C-2-substituted mannose respectively, in addition to the three *O*-methyl derivatives described above.

Isolation of oligosaccharides from unit A-type glycopeptides

Endo-β-*N*-acetylglucosaminidase H was shown to cleave the *NN'*-diacetylchitobiose linkage in the core of most high-mannose-type glycopeptides (Tarentino

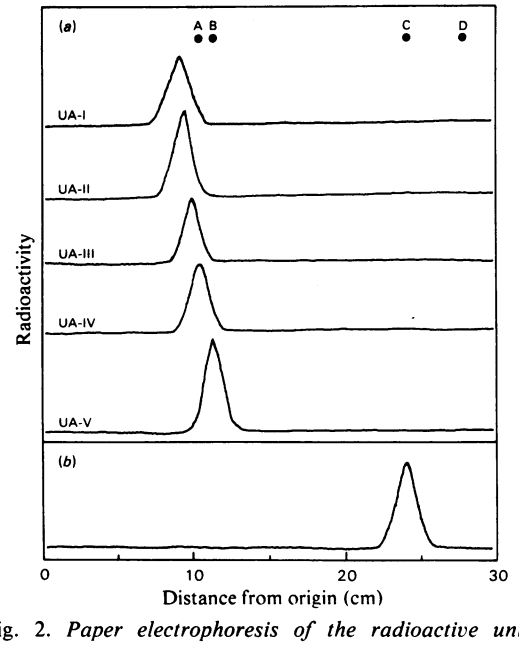

Fig. 2. *Paper electrophoresis of the radioactive unit A-type glycopeptides*

Experimental details are in the text. *N*-[^{14}C]-Acetylated fractions UA-I–UA-V (*a*) and their digest with endo-β-*N*-acetylglucosaminidase H (*b*) were subjected to paper electrophoresis in pyridine/acetate buffer at a potential of 70v/cm for 1.5h. A, B and C indicate the electrophoretic positions of standard glycopeptides: A, ovalbumin glycopeptide IV; B, ovalbumin glycopeptide V; C, GlcNAc-*N*-[^{14}C]acetyl-Asn. D indicates the position of Bromophenol Blue.

Table 1. *Chemical compositions of unit A-type glycopeptides from porcine thyroglobulin*
The fractions are as shown in Fig. 2. Molar ratios are expressed in relation to aspartic acid taken as 1.0.

Constituent	Fraction ...	Molar ratio UA-I	UA-II	UA-III	UA-IV	UA-V
Aspartic acid		1.0	1.0	1.0	1.0	1.0
Serine		0.1	—	—	—	—
Glutamic acid		0.1	0.1	0.1	—	—
Glycine		0.1	0.1	0.2	0.1	—
Alanine		—	—	0.1	—	—
Valine		—	—	0.1	—	—
Glucosamine		2.0	1.8	2.0	2.0	2.0
Mannose		9.0	7.7	7.2	6.1	5.0

& Maley, 1974; Arakawa & Muramatsu, 1974). The susceptibility of each unit A-type glycopeptide to this enzyme was examined by using the *N*-[^{14}C]-acetylated glycopeptide as a substrate. As shown in Fig. 2, *N*-[^{14}C]acetylated fractions UA-I–UA-V were completely converted by endo-β-*N*-acetylglucosaminidase H digestion into a radioactive peak with the same electrophoretic mobility as GlcNAc-*N*-[^{14}C]acetyl-Asn prepared from ovalbumin glycopeptide V. To obtain the oligosaccharides from unit A-type glycopeptides, larger quantities of unlabelled fractions UA-I–UA-V were digested with the enzyme. The released oligosaccharides were reduced with $NaBH_4$ or NaB^3H_4 (sp. radioactivity 50 Ci/mol; New England Nuclear, Boston, MA, U.S.A.) by the method of Takasaki & Kobata (1978), and subjected to gel-permeation chromatography on a Bio-Gel P-4 column. As shown in Fig. 3, each glycopeptide gave a single peak of an oligosaccharide alcohol (oligosaccharides I–V in Fig. 3), and the elution positions of oligosaccharides IV and V were found to be identical with those of $(Man)_6$-GlcNAcol and $(Man)_5$-GlcNAcol prepared from ovalbumin glycopeptides IV and V respectively. Oligosaccharides I–V were identified as $(Man)_9$-GlcNAcol, $(Man)_8$-GlcNAcol, $(Man)_7$GlcNAcol, $(Man)_6$-GlcNAcol and $(Man)_5$-GlcNAcol respectively, on the basis of the patterns of their elution from a Bio-Gel P-4 column, compositional analyses (Table 1) and methylation analyses (Table 2).

Exoglycosidase digestions of released oligosaccharides

To determine the sequence of each released oligosaccharide, the radioactive samples were digested first with α-mannosidase and then with β-mannosidase. The products of glycosidase digestions were chromatographed on a column of Bio-Gel P-4. All the oligosaccharides I–V, digested with α-mannosidase, gave a single radioactive product that co-migrated with Manβ1–4GlcNAcol. This radioactive peak was converted into *N*-acetylglucosaminitol by subsequent digestion with β-mannosidase. These results indicated that the

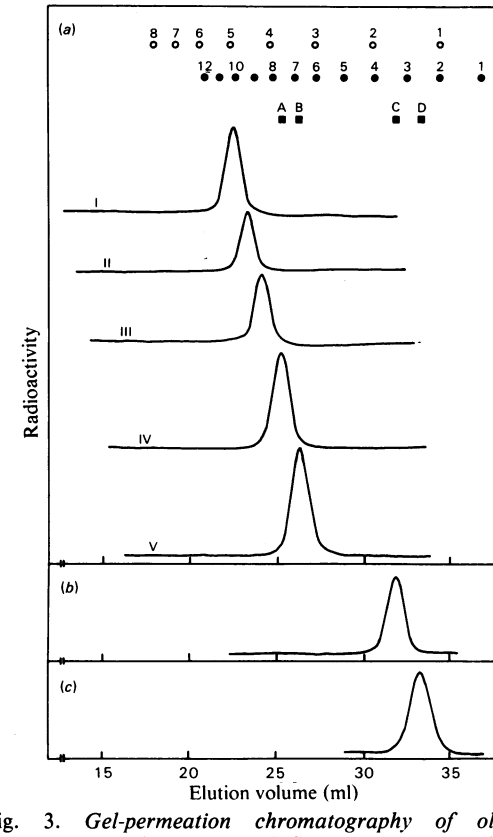

Fig. 3. *Gel-permeation chromatography of oligosaccharide alcohols obtained from unit A-type glycopeptides on a Bio-Gel P-4 column*
Experimental details are in the text. (*a*) The radioactive oligosaccharide alcohols released by endo-β-*N*-acetylglucosaminidase H digestion followed by reduction with NaB^3H_4; (*b*) product of α-mannosidase treatment of (*a*); (*c*) product of β-mannosidase treatment of (*b*). Closed circles (1–12), open circles (1–8) and closed squares (A–D) indicate the elution positions of standard sugars: ●, glucose oligomers; ○, *N*-acetylglucosamine oligomers; ■, oligosaccharide alcohols prepared from ovalbumin glycopeptides: A, $(Man)_6$-GlcNAcol; B, $(Man)_5$-GlcNAcol; C, Man-GlcNAcol; D, GlcNAcol.

Table 2. *Methylation analyses of unit A-type glycopeptides from porcine thyroglobulin*
The fractions are as shown in Fig. 1. Molar ratios are expressed in relation to 3,6-di-*O*-methylglucosaminitol as 2.0.

Sugars	*O*-Methyl group: Number	*O*-Methyl group: Position	Fraction ...	Molar ratio: UA-I	UA-II	UA-III	UA-IV	UA-V
Mannitol	4	2,3,4,6		3.0	3.3	3.3	3.1	3.0
	3	3,4,6		3.6	2.8	1.9	1.0	0.0
	2	2,4		2.1	2.2	2.1	1.9	1.8
2-Deoxy-2-*N*-methylacetamidoglucitol	2	3,6		2.0	2.0	2.0	2.0	2.0

Table 3. *Methylation analyses of the products of Smith periodate degradation or acetolysis of oligosaccharides I–V*
The fractions are as shown in Fig. 4. Molar ratios are expressed in relation to 2,3,4,6-tetra-*O*-methylmannitol taken as 1.0.

Sugars	*O*-Methyl group: Number	*O*-Methyl group: Position	Smith degradation fragment	Fraction ...	Molar ratio: Ac-1	Ac-2	Ac-3	Ac-4	Ac-5
Mannitol	4	2,3,4,6	1.0		1.0	1.0	1.0	1.0	1.0
	3	3,4,6	0.0		1.7	1.9	1.0	1.0	0.0
		2,4,6	0.0		1.1	1.0	1.0	1.1	1.3
		2,3,4	1.1		0.0	0.0	0.0	0.0	0.0
2-Deoxy-2-*N*-methyl-acetamidoglucitol	4	1,3,5,6	0.0		1.1	0.8	1.1	0.9	1.2
2-Deoxy-2-*N*-methyl-acetamidoxylitol	3	1,3,5	1.0		0.0	0.0	0.0	0.0	0.0

sequences of oligosaccharides I–V were $(\alpha Man)_{8-4}$-βMan-GlcNAcol.

Core structure of oligosaccharides I–V

The results of the methylation study (Table 2) and of enzymic digestion suggested that all five glycopeptides contained a common structure of Manα1→6(Manα1→3)Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc-Asn. Therefore, oligosaccharides I–V reduced with $NaBH_4$ were subjected to Smith periodate degradation to elucidate the branching pattern of mannose residues. When the products were analysed by gel-permeation chromatography and monitored by absorbance at 210nm, each oligosaccharide gave a single peak whose elution position was identical with that of Manα1→6Manβ1→4XylNAcol prepared from ovalbumin glycopeptide V (Tai *et al.*, 1975) by endo-β-*N*-acetylglucosaminidase H digestion followed by reduction with $NaBH_4$ and Smith periodate degradation (Fig. 4*a*). When the isolated products from oligosaccharides I–V were pooled and subjected to methylation analysis, 2,3,4,6-tetra-*O*-methylmannitol, 2,3,4-tri-*O*-methylmannitol and 1,3,5-tri-*O*-methylxylosaminitol were detected in a molar ratio of 1.0:1.1:1.0 (Table 3), indicating that this product had the structure of Manα1→6Manβ1→4XylNAcol. All five oligosaccharides contain, therefore, a $(Man)_5$GlcNAcol core with the structure of Manα1→6(Manα1→3)Manα1→6(Manα1→3)-Manβ1→4GlcNAcol. This structure was found to be the whole structure of oligosaccharide V.

Structures of oligosaccharides I–V

The compositional analysis (Table 1) and the methylation study (Table 2) indicated that oligosaccharides I–IV contained 4, 3, 2 and 1 more α1→2-linked mannose residues than oligosaccharide V respectively. The positions of the remaining mannose residues were determined by subjecting the oligosaccharide alcohols reduced with $NaBH_4$ to acetolysis, a procedure that preferentially cleaves Manα1→6Man linkages. The resulting fragments were fractionated by gel-permeation chromatography on a Bio-Gel P-4 column after reduction with NaB^2H_4, and the elution was monitored by absorbance at 210nm for oligosaccharides containing *N*-acetylglucosaminitol and by refractive index for whole sugars.

Oligosaccharide I. The major product (Ac-1 in Fig. 4*b*) containing *N*-acetylglucosaminitol, observed after acetolysis of oligosaccharide I, was identified as Manα1→2Manα1→2Manα1→3Manβ1→4GlcNAcol on the basis of the results of methylation studies (Table 3) and the Smith periodate degradation experiment. In addition to the oligosaccharide containing *N*-acetylglucosaminitol, two fragments corresponding to trisaccharide alditol (tri-itol) and disaccharide alditol (di-itol) were detected (Fig. 4*b*). Methylation analysis revealed

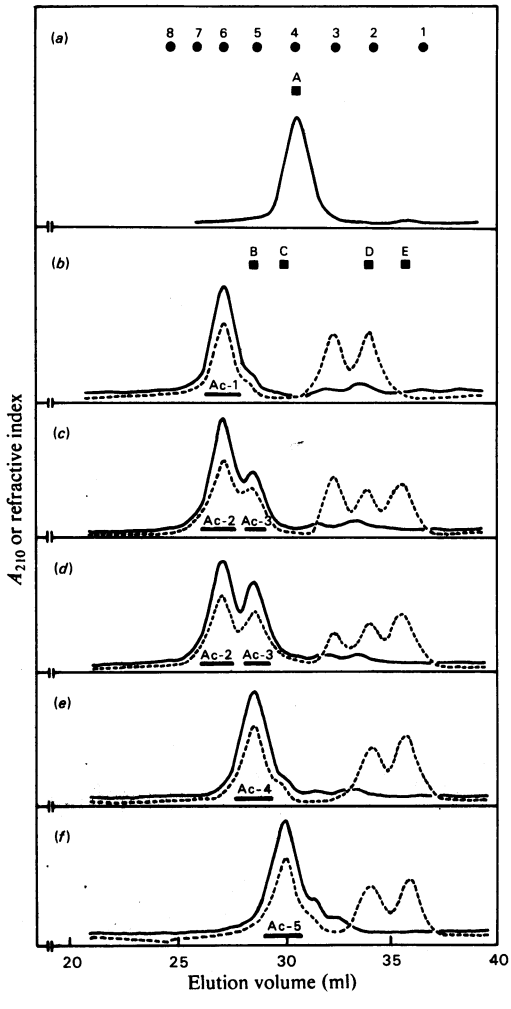

Fig. 4. *Gel-permeation chromatography of the products of Smith periodate degradation (a) and acetolysis (b–f) of oligosaccharides I–V on a Bio-Gel P-4 column*
Experimental details are in the text. (*a*) Smith periodate degradation products of oligosaccharides I–V; (*b*) acetolysate of oligosaccharide I; (*c*) acetolysate of oligosaccharide II; (*d*) acetolysate of oligosaccharide III; (*e*) acetolysate of oligosaccharide IV; (*f*) acetolysate of oligosaccharide V. Closed circles (1–8) and squares (A–E) indicate the elution positions of standard sugars: ●, glucose oligomers; ■, oligosaccharide alcohols prepared from ovalbumin glycopeptides; A, Manα1→6Manβ1→4XylNAcol; B, Manα1→2Manα1→3Manβ1→4GlcNAcol; C, Manα1→3Manβ1→4GlcNAcol; D, Manα1→3mannitol; E, mannitol. Elution was carried out with water at a flow rate of 0.3 ml/min and monitored by A_{210} (——) for oligosaccharide alcohols containing GlcNAcol or XylNAcol and by refractive index (– – – –) for whole sugars.

that 1,2,4,5,6-penta-*O*-methylmannitol, 2,3,4,6-tetra-*O*-methylmannitol and 3,4,6-tri-*O*-methylmannitol were derived from the tri-itol, and from the di-itol 1,3,4,5,6-penta-*O*-methylmannitol and 2,3,4,6-tetra-*O*-methylmannitol were derived. On the basis of these results the tri-itol and the di-itol were identified as Manα1→2Man(α1→3)mannitol and Man(α1→2)-mannitol respectively.

This acetolysis pattern indicates that the structure of oligosaccharide I is Manα1→2Manα1→6(Manα1→2Manα1→3)Manα1→6(Manα1→2Manα1→2Manα-1→3)Manβ1→4GlcNAcol.

Oligosaccharide II. Two peaks of oligosaccharide alcohols containing *N*-acetylglucosaminitol were obtained from the acetolysate of oligosaccharide II in a molar ratio of 7:3 (Fig. 4*c*). One of these peaks eluted at the same position as Manα1→2Manα1→2Manα1→3Manβ1→4GlcNAcol derived from oligosaccharide I, and the other one, which was smaller in size by one mannose residue, eluted at the same position as authentic Manα1→2Manα1→3Manβ1→4GlcNAcol prepared from ovalbumin glycopeptide IV. The two products were identified as Manα1→2Manα1→2Manα1→3Manβ1→4GlcNAcol and Manα1→2Manα1→3Manβ1→4GlcNAcol by the methylation analysis (Table 3). Three fractions that did not contain *N*-acetylglucosaminitol were also obtained at the elution positions of tri-itol, di-itol and mannitol (Fig. 4*c*). Methylation analysis showed that the tri-itol was Manα1→2Man(α1→3)mannitol and the di-itol was a mixture of Man(α1→2)mannitol and Man(α1→3)mannitol in a molar ratio of 5:3. Since 1,3,4,5,6-penta-*O*-methylmannitol and 1,2,4,5,6-penta-*O*-methylmannitol could not be separated completely on a column of 2% OV-17, the ratio of these derivatives is approximate. These results, therefore, indicate that oligosaccharide II is a mixture of Manα1→2Manα1→6(Manα1→2Manα-1→3)Manα1→6(Manα1→2Manα1→3)Manβ1→4-GlcNAcol (approx. 30%), Manα1→2Manα1→6-(Manα1→3)Manα1→6(Manα1→2Manα1→2Manα-1→3)Manβ1→4GlcNAcol (approx. 45%) and Manα-1→6(Manα1→2Manα1→3)Manα1→6(Manα1→2-Manα1→2Manα1→3)Manβ1→4GlcNAcol (approx. 25%).

Oligosaccharide III. Manα1→2Manα1→2-Manα1→3Manβ1→4GlcNAcol and Manα1→2-Manα1→3Manβ1→4GlcNAcol were detected in a molar ratio of 3:2 in the acetolysate of oligosaccharide III (Fig. 4*d*). The tri-itol was identified as Manα1→2Manα1→3mannitol since methylation analysis revealed that this fragment consisted of 2,3,4,6-tetra-*O*-methylmannitol, 3,4,6-tri-*O*-methylmannitol and 1,2,4,5,6-penta-*O*-methylmannitol. The di-itol fraction was found to be a mixture of Man(α1→3)mannitol and Man(α1→2)mannitol in a molar ratio of approx. 4:1. These results indicate

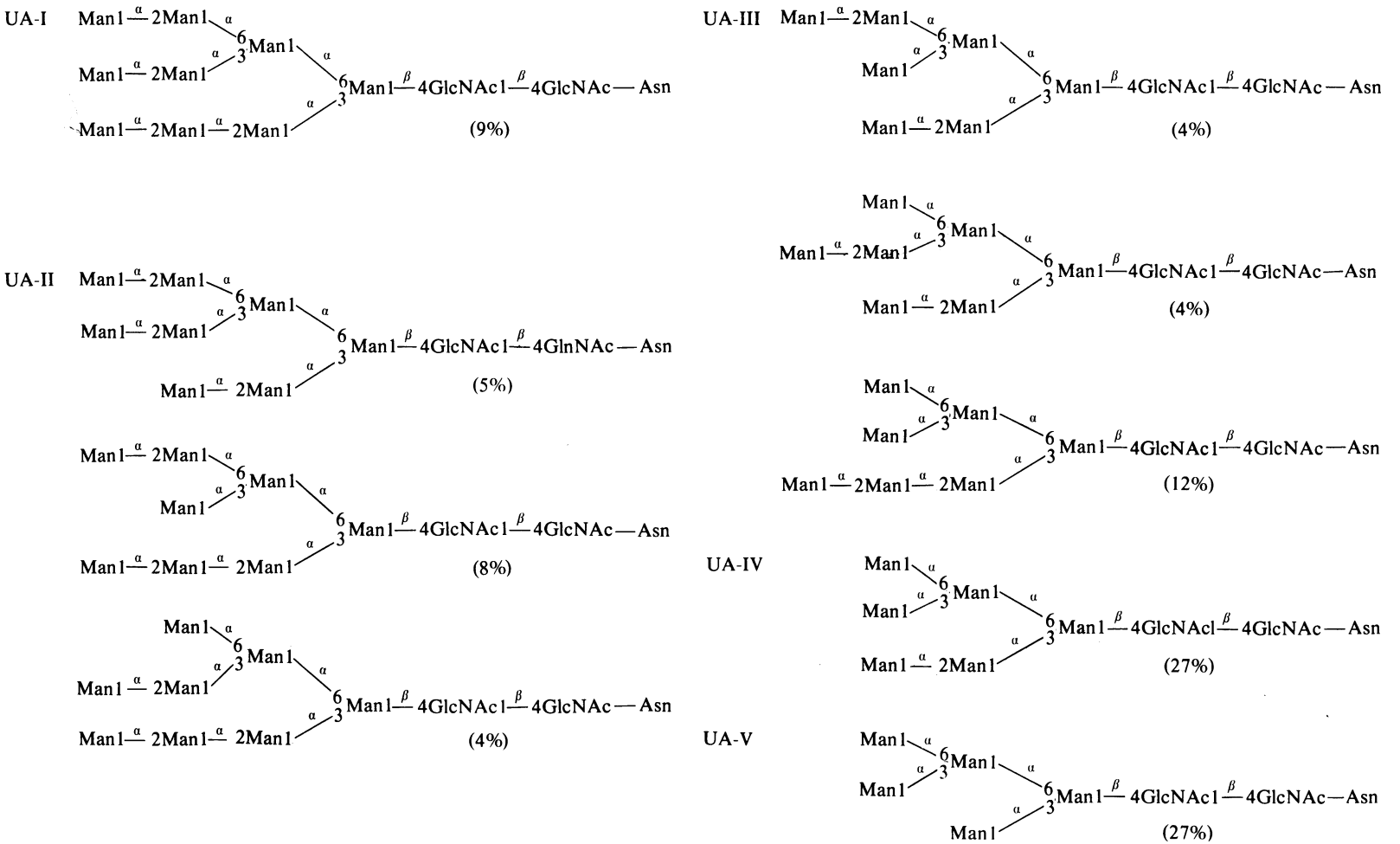

Fig. 5. *Proposed structures for unit A-type glycopeptides from porcine thyroglobulin*

Values in parentheses represent the amounts of respective glycopeptides present expressed as a percentage of the total unit A-type glycopeptides, which were obtained by Dowex 50 W (X2) column chromatography.

that oligosaccharide III is a mixture of Manα1→2Manα1→6(Manα1→3)Manα1→6(Manα1→2Manα1→3)Manβ1→4GlcNAcol (approx. 20%) Manα1→6(Manα1→2Manα1→3)Manα1→6(Manα1→2Manα1→3)Manβ1→4GlcNAcol approx. 20%) and Manα1→6(Manα1→3)Manα1→6(Manα1→2Manα1→2Manα1→3)Manβ1→4GlcNAcol (approx. 60%).

Oligosaccharide IV. The acetolysate of oligosaccharide IV contained a single product that eluted at the same position as authentic Manα1→2Manα1→3Manβ1→4GlcNAcol prepared from ovalbumin glycopeptide IV, when sugars were monitored by absorbance at 210nm (Fig. 4*e*). Methylation analysis (Table 3), together with the Smith periodate degradation experiment, established the structure of the product to be Manα1→2Manα1→3Manβ1→4GlcNAcol. This indicates that the structure of oligosaccharide IV is Manα1→6(Manα1→3)Manα1→6(Manα1→2Manα1→3)-Manβ1→4GlcNAcol.

Oligosaccharide V. The Smith periodate degradation experiment and methylation analysis indicated that the structure of oligosaccharide V is Manα1→6(Manα1→3)Manα1→6(Manα1→3)-Manβ1→4GlcNAcol as described above. This was confirmed by an acetolysis experiment. The acetolysate of oligosaccharide V gave rise to Manα1→3Manβ1→4GlcNAcol (Fig. 4*f*, Table 3) as the sole product containing *N*-acetylglucosaminitol.

Proposed structures of unit A-type glycopeptides from porcine thyroglobulin

Based on the results so far presented and from the specificity of endo-β-*N*-acetylglucosaminidase H, the complete structures of glycopeptides UA-I–UA-V from porcine thyroglobulin are proposed to be as shown in Fig. 5.

Discussion

The structures of unit A-type glycopeptides from porcine thyroglobulin are shown in Fig. 5. All contain a $(Man)_5(GlcNAc)_2$-Asn unit identical with the structure of glycopeptide UA-V as a core structure. They differ only in the number of outer mannose residues α(1→2)-linked to the $(Man)_5(GlcNAc)_2$-Asn core. Compared with the structure of processing intermediates in the biosynthetic pathway of asparagine-linked sugar chains (Kornfeld *et al.*, 1978), unit A-type glycopeptides appear to be precursors that are to be processed to form unit B-type sugar chains. Glycopeptide UA-I, whose structure is identical with that of the major unit A-type glycopeptide from calf thyroglobulin (Ito *et al.*, 1977), has an oligosaccharide the same as a lipid-linked oligosaccharide reported by Li *et al.* (1978), except for the absence of glucose residues in this glycopeptide. Glycopeptides UA-II–UA-V seem to be the biosynthetic products of glycopeptide UA-I in the process of stepwise removal of α(1→2)-linked mannose residues. This removal, which is probably catalysed by α-mannosidase (Tabas & Kornfeld, 1979) in the porcine thyroid gland, appears to take place randomly, since both fractions UA-II and UA-III are composed of three respective isomers. This observation is in agreement with those for bovine pancreatic ribonuclease B (Liang *et al.*, 1980) and urinary oligosaccharides from α-mannosidosis patients (Yamashita *et al.*, 1980), but is different from those for the cases of human immunoglobulin M myeloma protein (Chapman & Kornfeld, 1979) and Chinese hamster ovary cell glycoprotein (Li & Kornfeld, 1979). However, among α(1→2) linkages between mannose residues, the inner one in the branch arising from C-3 of β-linked mannose appears to be rather resistant to this α-mannosidase, since in glycopeptides UA-III and UA-IV this linkage remained unhydrolysed. Especially in fraction UA-IV, this remained as the only α(1→2) linkage. One possibility is that the α-mannosidase involved in this processing step has a different specificity of activity among the tissues or the species.

Structural studies of ovalbumin glycopeptides have been extensively carried out by Kobata and co-workers (Tai *et al.*, 1975, 1977; Yamashita *et al.*, 1978), and the results have demonstrated that the glycoprotein contained both high-mannose-type and hybrid-type sugar chains. On the other hand, it is noteworthy that porcine thyroglobulin has no hybrid-type sugar chains. This suggests that the subsequent processing system of the biosynthesis of asparagine-linked sugar chains is quite specific in each tissue. The glycopeptides and oligosaccharides from porcine thyroglobulin (unit A and unit B), which can be prepared rather easily on a large scale, are good standards for structural and functional studies of various glycoproteins.

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